EVIDENCE FOR A STEREOSPECIFIC [^{3}H]ETORPHINE BINDING IN HUMAN PLACENTA

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1. Introduction

Placental tissue is a source of receptors for some polypeptidic hormones including insulin [1] and somatomedin [2]. In addition it produces some chorionic, pituitary-like hormones, such as gonadotropin [3], somatomammotropin [4], corticotropin [5]. A recent report [6] indicates that human full term placenta contains β-lipotropin and β-endorphin. β-Endorphin is a polypeptide with morphine-like properties that exhibits a high affinity for opiate receptors in brain and peripheral tissues [7–9]. Evidence will be presented here that human placenta contains substantial amounts of stereospecific binding sites for [^{3}H]etorphine, a potent opiate agonist.

2. Materials and methods

2.1. *Preparation of human placental membranes*

Placentae were collected on ice immediately after caesarian section, minced with scissors and washed several times with cold physiological serum. All further operations were carried out at 4°C. The tissue was freed from blood vessels and connective tissue, and homogenized in 5 vol. Tris–HCl buffer 0.05 M at pH 7.4 in a Potter Elvehjem (5 strokes). The homogenate was centrifuged (1000 × *g* for 10 min) and the supernatant fluid was spun at 100 000 × *g* for 30 min; the resulting pellet was washed once and spun again for an additional 30 min at 100 000 × *g*. The final pellet was homogenized, then diluted with buffer to give ~ 1 mg protein/ml final conc.

2.2. *Binding assay*

The reaction mixtures (1 ml) contained 0.8 mg protein, 1 pmol [^{3}H]etorphine with or without 1 nmol unlabelled etorphine. They were incubated, always in quadruplicate, at 37°C for 30 min. Immediately after incubation, they were filtered under reduced pressure through Whatman glass fibre disks (GFB) and washed with 10 ml of ice-cold Tris buffer. The filters were dried and counted in 10 ml toluene scintillation cocktail. Protein concentrations were estimated by the method in [10]. Specific [^{3}H]etorphine binding was defined as the difference between the radioactivity bound to membranes in the absence and in the presence of 1 μM of non-radioactive etorphine.

2.3. *Chemicals*

[15,16(*n*)-^{3}H]etorphine, 35.4 Ci/mmol, the Radiochemical Center, Amersham; Dextrorphan and Levorphanol. Hoffman-La Roche, Basel; Naloxone, Endo, Brussels; Morphine, Francopia, Paris; D-Ala–Leu-enkephalinamide, Dr Mazarguil, Toulouse.

3. Results

The time course of [^{3}H]etorphine binding to placental membranes is shown in fig.1. At 37°C specific binding of the tritiated ligand (1 nM) reached equilibrium within ~ 10 min and remained constant at least up to 40 min.

Reversibility of [^{3}H]etorphine binding was demonstrated by the addition of 10^{-5} M unlabelled etorphine which resulted in a complete dissociation

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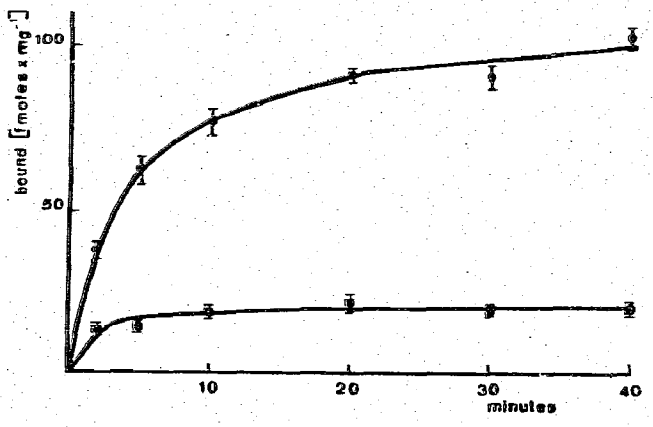

Fig.1. [^{3}H]Etorphine binding to human placental membranes as a function of time. [^{3}H]Etorphine (10^{-9} M) was incubated with placental membranes (1 mg protein/ml) for the indicated periods of time at 37°C with (■) or without unlabelled etorphine 10^{-6} M; the specific [^{3}H]etorphine binding (●) was determined as in section 2.

of the specifically labelled complex within 10 min at 37°C. Analysis of the kinetics studies represented in fig.2 indicated an association constant K_1 of 0.19×10^9 M^{-1} min^{-1} and a dissociation rate constant K_{-1} of 0.075 min^{-1}.

Specific binding of [^{3}H]etorphine (in the range $0.125–4 \times 10^{-9}$ M) to the placental membrane fragments was saturable. A Scatchard analysis indicated a single class of non-interacting binding sites with a K_d of 0.59 ± 0.13 10^{-9} M (mean ± SEM, $n = 5$) and a binding capacity of ~ 70 fmol/mg protein. One experiment is shown in fig.3. The K_d value, derived from the Scatchard plot (0.59×10^{-9} M) agreed very well with the K_d value of 0.39×10^{-9} M calculated from the ratio K_{-1}/K_1.

We have measured the K_d and the capacity of [^{3}H]etorphine binding site on the membranes of two placentae obtained from early pregnancies. The values obtained were K_d 0.45 nM, capacity 44 fmol/mg

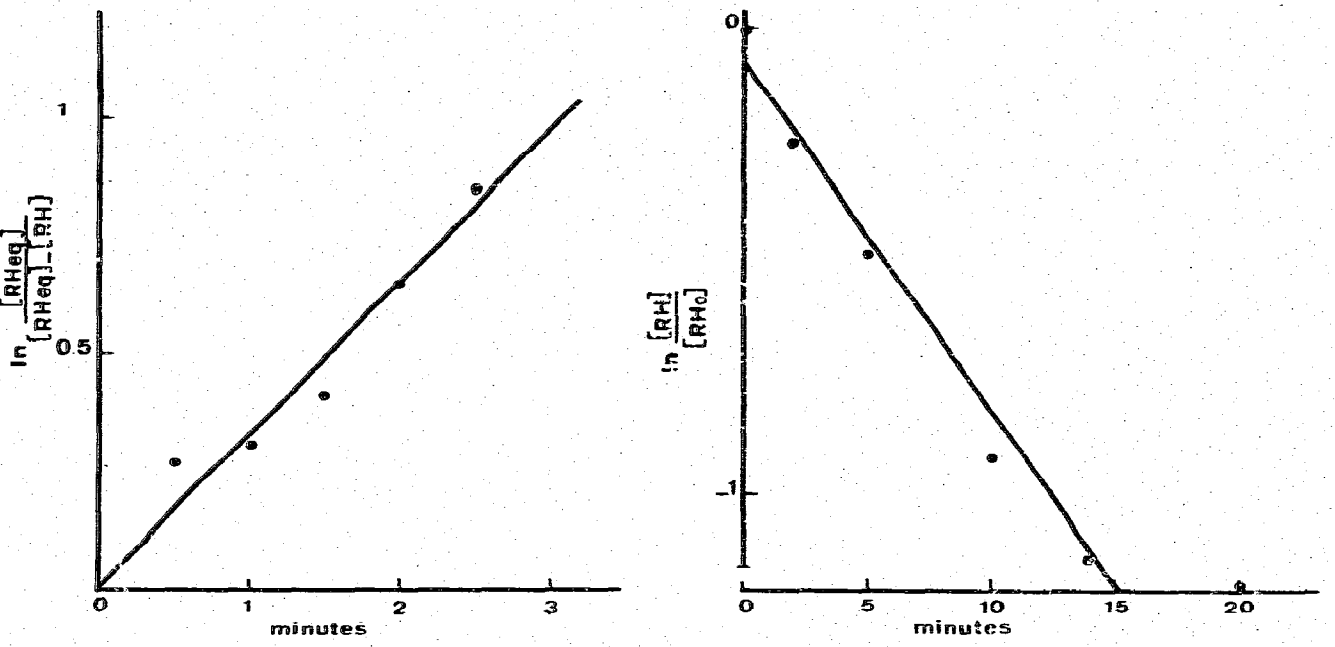

Fig.2. Determination of rate constants. Analysis of association (left panel): [RHeq], concentration of the complex [^{3}H]etorphine–membrane at equilibrium; [RH], concentration of the complex [^{3}H]etorphine–membrane at a given time. The line determined by linear regression analysis ($r = 0.98$) has a slope of K ob., K_1 is calculated from $K_1 = K$ ob. $-K_{-1}$/[etorphine] where K_{-1} is the first order rate constant for dissociation and [etorphine] the concentration of [^{3}]etorphine in the assay (1.25 nM). Reversibility of [^{3}H]etorphine binding to placental membranes (right panel). Membranes were incubated with [^{3}H]etorphine at 37°C for 20 min after which a large excess of etorphine (10^{-5} M) was added. [RH], concentration of the complex [^{3}H]etorphine–membrane at a given time; [RH]o, concentration of the complex at 0 time of the dissociation studies. The line determined by linear regression analysis ($r = 0.99$) has a slope (K_{-1}) of 0.075 min^{-1}.

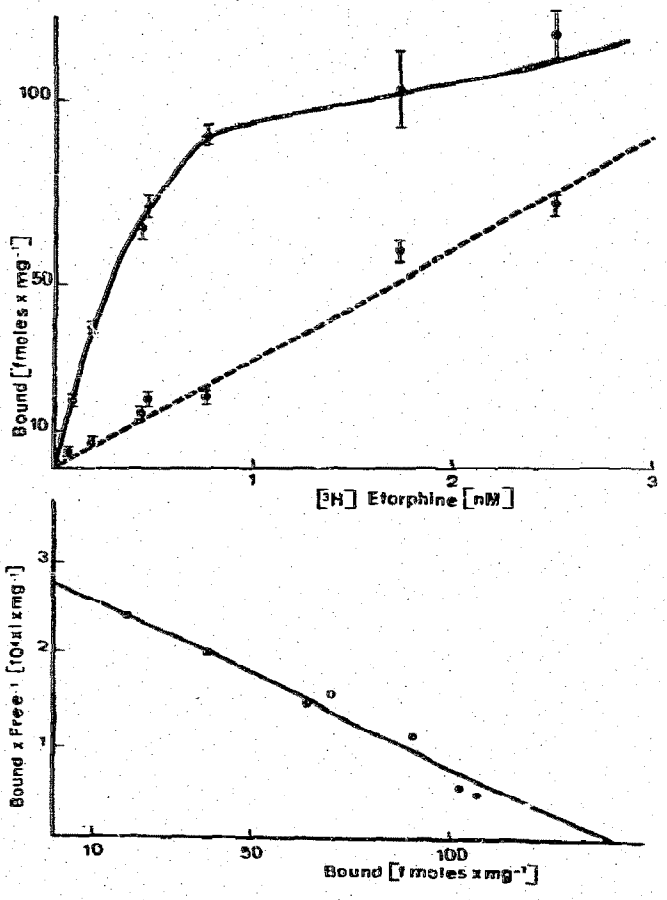

Fig.3. Binding of [^{3}H]etorphine to placental membranes as a function of [^{3}H]etorphine concentration. Non-specific binding (—) is measured with 1 μM labelled etorphine; specific binding (——) is measured as described in section 2.2. Each value is the mean ± SEM of 4 determinations. The experiment shown is representative of 5 such experiments (upper panel). Scatchard plot of specific [^{3}H]etorphine binding to human placental membranes is shown in the lower panel.

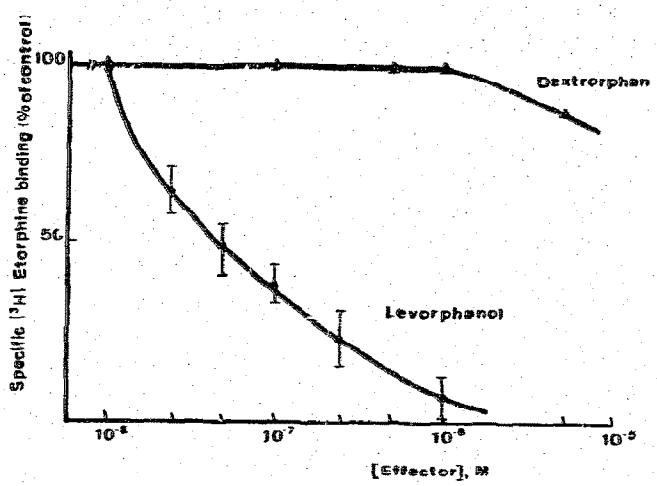

Fig.4. Stereoselective competition for [^{3}H]etorphine binding. [^{3}H]Etorphine (1 nM) was incubated with a placental membranes fraction (1 mg protein/ml) with the indicated concentrations of levorphanol (●) or dextrorphan (▲). Binding inhibition is expressed as percent of specific [^{3}H]etorphine binding measured as described in section 2.2. Each value is the mean ± SEM of 4 determinations from 3 expt.

protein and K_d 0.85 nM, capacity 80 fmol/mg protein for respectively 4 and 5 months of gestation.

Figure 4 shows that [^{3}H]etorphine binding to placental membranes is stereospecific as defined [11] since it was prevented by levorphanol but not by its inactive enantiomorph dextrorphan.

The specificity of [^{3}H]etorphine binding was demonstrated by the inhibitory effect of various compounds (table 1). The K_i values for levorphanol and naloxone were similar with the affinities of these compounds for brain opiate receptors as reported [12]. Morphine was 10-fold less potent in reducing binding of [^{3}H]etorphine to placental membranes than to brain membranes [12]. The opioid peptide D Ala–Leu-enkephalinamide was able to compete with [^{3}H]etorphine for binding to placental membranes.

Addition of sodium salt has been reported to enhance stereospecific binding of antagonist and to decrease those of agonist to rat brain homogenate [13]. We have measured the binding of 1 nM [^{3}H]etor-

Table 1
Competition of drugs for specific binding of [^{3}H]etorphine to human placental membranes

Drugs	K_i (nM)
Levorphanol	18.5
Dextrorphan	> 10 000
Naloxone	11
Morphine	2000
D-Ala–Leu-enkephalinamide	850

[^{3}H]Etorphine (1 nM) was used. IC_{50} values from 3–6 expt were determined from log probit plots and converted to K_i values according to the formula: $K_i = IC_{50}/(1 + C/K_d)$, where C is the concentration and K_d the affinity of [^{3}H]etorphine binding site

phine to placental membranes in the presence and in the absence of 160 mM NaCl. The stereospecific binding of [^{3}H]etorphine was reduced by 75 ± 5% (mean ± SEM, $n = 3$).

4. Discussion

This study has shown that [^{3}H]etorphine binds reversibly and with a high affinity (K_d 0.6 nM) to saturable sites in a membrane fraction of human placenta. This binding is stereospecific because it is not inhibited by dextrorphan (at $> 10^{-5}$ M) but is maximally inhibited by 10^{-6} M levorphanol. Morphine, naloxone and D-Ala–Leu-enkephalinamide are able to compete with [^{3}H]etorphine for binding to placental membranes: the K_i values for naloxone and levorphanol are similar to those measured for these compounds in brain [12]. The very low affinity of morphine for [^{3}H]etorphine binding sites might result from metabolic inactivation of the drug by the placental microsomes present in our membrane fraction. In fact, the placenta of many species, including man, is known to exhibit drug metabolizing activity [14]. Stereospecific binding of [^{3}H]etorphine to placental membranes is reduced in the presence of NaCl. Thus [^{3}H]etorphine binding to sites from placental membranes have several characteristics in common with opiate receptors from other tissues [12,13].

It seems likely that the ability of human placenta to bind opiates stereospecifically exists early in pregnancy and presents properties analogous to those described in human full-term placenta.

Placental tissue is devoid of innervation so that the biological significance of its opiate binding sites remains to be elucidated.

Acknowledgements

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References

[1] Posner, B. I. and Montreal, M. D. (1974) Diabetes 23, 209–217.
[2] Marshall, R. N., Underwood, L. E., Voina, S. J., Founshee, D. B. and Van Wyk, J. J. (1974) J. Clin. Metab. 39, 283–292.
[3] Midgley, A. R. and Pierce, G. B. (1962) J. Exp. Med. 115, 289–294.
[4] Josimovich, J. B. and Mac Laren, J. A. (1962) Endocrinology 71, 209.
[5] Rees, L. H., Burke, C. W., Chard, T., Evans, S. W. and Letchworth, A. T. (1975) Nature 254, 620–622.
[6] Nakai, Y., Nakao, K., Oki, S. and Imura, H. (1978) Life Sci. 23, 2013–2018.
[7] Li, C. H. and Chung, D. (1976) Proc. Natl. Acad. Sci. USA 73, 1145–1148.
[8] Loh, H. H., Tseng, L. F., Wei, E. and Li, C. H. (1976) Proc. Natl. Acad. Sci. USA 73, 2895–2898.
[9] Lazarus, L. H., Ling, N. and Guillemin, R. (1976) Proc. Natl. Acad. Sci. USA 73, 2156–2159.
[10] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
[11] Goldstein, A., Lowney, L. I. and Pal, B. K. (1971) Proc. Natl. Acad. Sci. USA 68, 1742–1747.
[12] Simon, E. J., Hiller, J. M. and Edelman, I. (1973) Proc. Natl. Acad. Sci. USA 70, 1947–1949.
[13] Simon, E. J., Hiller, J. M., Groth, J. and Edelman, I. (1975) J. Pharmacol. Exp. Ther. 192, 531–537.
[14] Juchau, M. R., Niswander, K. B. and Yaffe, S. J. (1968) Am. J. Obstet. Gynecol. 100, 348–357.